Nutrition and Vitamin B₁₂ Metabolism of a Soil Bacterium

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SUMMARY

The growth requirements of a vitamin B₁₂-dependent soil bacterium (strain 544), provisionally classified as belonging to the genus *Arthrobacter*, were studied. The organism showed a high specificity for vitamin B₁₂; of the naturally occurring analogues tested, only α -(5-hydroxybenzimidazolyl)-cobamide cyanide and α -(2-methylmercaptoadenyl)cobamide cyanide possessed some growth-promoting activity (less than 10%). The anilide and the ethylamide of the monocarboxylic acid of vitamin B₁₂ inhibited growth and the inhibition was reversed by the vitamin. A medium and a procedure for vitamin B₁₂ assay using strain 544, is described. Comparative assays with *Ochromonas malhamensis* and *Escherichia coli* 7M are given and their differential usefulness for differentiating the various congeners of vitamin B₁₂ discussed.

Addition of vitamin B₁₂ to B₁₂-deficient cell suspensions of strain 544 stimulated the biosynthesis of methionine and the oxidation of propionate, valerate, isoleucine and valine. The endogenous respiration and oxidation of glucose, butyrate, leucine and lysine were only slightly enhanced by vitamin B₁₂.

INTRODUCTION

Lochhead and co-workers have shown that the soil is an important habitat of vitamin B₁₂-requiring bacteria (Lochhead & Thexton, 1951, 1952; Lochhead & Burton, 1956, 1957). In a detailed study of vitamin B₁₂-requiring bacteria obtained from field soil by a 'non-selective' procedure, the majority of the isolates were considered to belong to the genus *Arthrobacter* (Lochhead & Burton, 1955). Ford & Hutner (1957), who studied the nature of the vitamin B₁₂ requirement of a number of these isolates, found that one strain responded to factor A, factor B and pseudovitamin B₁₂, while all the rest responded only to vitamin B₁₂ and vitamin B₁₂III and thus resembled *Ochromonas malhamensis* in their pattern of response to the various B₁₂ vitamins. The latter authors emphasized the promising features of several of these isolates as vitamin B₁₂ reagents. However, in a more detailed study of one of these organisms (Lochhead 38) Goldberg, Hutner & Ford (1957) found that although its response was limited to cyanocobalamin and vitamin B₁₂III, it was unsuitable for tube assays of the vitamin because of its granular and non-dispersible type of growth. Moreover Sanders & Seaman (1960) reported that 'Lochhead 38' also responded to α -(2-methyladenyl)cobamide cyanide, factor C and factor B.

This report describes the nutritional requirements and physiological characteristics of another of Lochhead's isolates, no. 544. The nature of the vitamin B₁₂ requirement of this organism was studied, and its specificity for the B₁₂ vitamins was compared to that of a B₁₂-requiring strain of *Escherichia coli* 7M and of *Ochromonas malhamensis*.

A growth medium and a procedure for a tube assay of vitamin B₁₂ using isolate 544 are described. Some metabolic effects of vitamin B₁₂ were studied using vitamin B₁₂-deficient suspensions of organisms.

METHODS

Micro-organisms used. Isolate no. 544—a soil bacterium received from Dr A. G. Lochhead; *Escherichia coli*, 7M, a vitamin B₁₂ or methionine-requiring mutant of *E. coli* W; *Ochromonas malhamensis* ATCC 11352.

Cobinamide and the benziminazol and adenine analogues of vitamin B₁₂ were kindly supplied by Professor K. Bernhauer. The anilide and ethylamide of the mono-carboxylic acids of vitamin B₁₂ were obtained through the courtesy of Dr E. Lester Smith. Factors Z₂, Z₃, which were isolated from digested sewage sludge, were kindly given by Dr Halina Neujahr. α -(5,6-dimethylbenzimidazolyl)cobamide cyanide (vitamin B₁₂) was purchased from Nutritional Biochemicals Co., U.S.A. All compounds were dissolved in water and standardized spectrophotometrically at 361 m μ using vitamin B₁₂ as standard. Cobinamide and factors Z₂ and Z₃ were standardized at 355 m μ .

Growth. *Escherichia coli* was grown in a glucose mineral medium (Davis & Mingioli, 1950). The quantitative assay of vitamin B₁₂ with this organism was done as described previously (Aronovitch & Grossowicz, 1958). *Ochromonas malhamensis* was grown in the dark according to the procedure and medium described by Ford (1953, 1958). The nutritional requirements of isolate 544 were studied by using the basal medium (Table 1), supplemented with vitamin B₁₂ (0.4 m μ g./ml.). Utilization of various energy sources was tested in the basal medium with citrate and Tween 80 omitted. Cultures were grown in test-tubes (19 $\times$ 150 mm.) containing 5 ml. medium, or in Erlenmeyer flasks (50 ml.) containing 10 ml. medium. A 48 hr culture was diluted 1/10 with sterile medium and one drop of this culture was used to inoculate each assay tube.

Assays of vitamin B₁₂ and related compounds were done in the complete medium (Table 1). The inoculum was grown for 48 hr on the same medium, supplemented with vitamin B₁₂ 0.1 m μ g./ml. One drop of this culture was used to inoculate each assay tube. The cultures were incubated at 28–30° on a reciprocal shaker (160–180 strokes/min.) for 48–72 hr. Growth was measured turbidimetrically in a Coleman nephometer at 590 m μ . Cultures giving a scale reading higher than 0.50 were diluted 1/3 with water before reading.

Manometric experiments. The organisms were grown for 48 hr in the complete medium containing a low concentration of ammonium nitrate (0.02 %) and no Tween 80. The medium was supplemented with Na succinate (0.4 %) and a suboptimal concentration of vitamin B₁₂ (0.1 m μ g./ml.). Organisms were collected by centrifugation, taken up in water and suspended in an equal volume of fresh medium without vitamin B₁₂, Casamino acids or energy source. The suspensions were aerated for 2 hr at 30° to decrease the endogenous reserves and then collected by centrifugation and washed once with water.

Oxygen consumption was measured in the conventional Warburg apparatus at 30°. Each vessel contained: suspension 1 ml., containing equiv. 4–6 mg. (dry wt organisms; phosphate buffer (pH 7.0), 10 μ moles; tris-HCl buffer (pH 7.0), 50 μ moles; substrate as indicated, 4 μ moles; vitamin B₁₂, 40 m μ g.; total volume, 2 ml. The centre well contained 0.1 ml. of 15 % KOH solution.

Media. The composition of the complete medium, vitamin B₁₂ omitted, is given in Table 1.

The basal medium was that described in Table 1 modified as follows: Casamino acids were omitted, and DL-methionine (20 µg./ml.) and trace elements added. Trace elements were supplied as sulphates to give final concentrations/l.: Zn²⁺, 0.25 mg.; Mn²⁺, 0.1 mg.; Cu²⁺, 0.02 mg./l. Vitamin B₁₂, when included, 0.4 mµg./ml.

Table 1. *Complete liquid medium for vitamin B₁₂ assays with Arthrobacter isolate 544*

The medium was adjusted to pH 6.8–7.0, boiled, filtered and sterilized by autoclaving for 15 min at 115°. The medium was usually prepared as a five-fold concentrated solution and stored at –20° in polyethylene bottles. Glucose (solution) was added aseptically to the medium after autoclaving, to give 0.4% (w/v) glucose.

Casamino acids (Difco) vitamin free	2.0 g	CaCO ₃	0.01 g
K ₂ HPO ₄	1.0 g	Ferrous ammonium sulphate	0.72 g
KH ₂ PO ₄	0.4 g	Tween 80	1.0 g
MgSO ₄ ·7H ₂ O	0.3 g	EDTA	0.005 g
Potassium citrate	0.5 g	Thiamine HCL	0.002 g
NH ₄ NO ₃	1.0 g	Distilled water to	1 l

RESULTS

Morphology and physiology of isolate 544

Young cultures of isolate 544 consisted of Gram-variable, non-sporeforming pleomorphic rods and some large coccoid forms. In older cultures, small coccoid forms, mostly Gram-negative, were predominant. Motility was not observed. Colonies, on suitable solid media, were small (1–2 mm in diameter), circular, convex, with smooth surface and of a soft consistency. On brain–heart infusion agar growth was abundant, with the production of a brownish water-soluble pigment. On nutrient agar there was no growth. In the complete solid medium, growth was colourless in young cultures and varied to pale brown in old ones. Acid was formed from glucose; starch was hydrolysed; nitrate was reduced to nitrite; indole was not formed; the organism was urease-negative and catalase-positive. Nitrate and ammonium were used as sole nitrogen sources. In the defined medium, vitamin B₁₂ and thiamine were required for growth. The organism was strongly aerobic. Good growth occurred between 20° and 30°; there was no growth at 37°. In accordance with these properties the strain was classified provisionally as belonging to the genus *Arthrobacter*.

Nitrogen sources. Utilization of various nitrogen sources for growth was tested by using the basal medium without methionine. Nitrogen compounds were added to a concentration of 0.1% (w/v). Isolate 544 grew well on nitrate, ammonium salts (sulphate, chloride, acetate) and casein hydrolysate as sole sources of nitrogen. Casein hydrolysate served as an energy source as well.

Energy sources. Utilization of different energy sources was tested by using the basal medium from which methionine and Tween 80 were omitted. Glucose, sucrose, succinate and glycerol were found to be suitable energy sources, yielding comparable growth rates; starch, glycogen, inulin, cellobiose and rhamnose were utilized more slowly; there was no growth on acetate, propionate, tartrate or citrate. Addition of Tween 80 (up to 0.2%) to the glucose-containing medium shortened the lag period and increased the total crop of organisms.

Trace elements. Growth on the basal medium was slow unless the medium was supplemented with trace amounts of Zn^{2+} , Mn^{2+} and Cu^{2+} . The salt mixture supplying these cations could be replaced by 15–20 % (v/v) boiled and filtered tap water. Addition of these trace elements proved unnecessary when casein hydrolysate was used as nitrogen source.

Autolysis. Cultures of isolate 544 grown in the complete medium without EDTA (Table 1) were prone to autolysis. Lysis usually began at the end of the exponential phase of growth, and became pronounced at suboptimal concentrations of vitamin B_{12} . The occurrence of autolysis interfered with the use of the isolate 544 for vitamin B_{12} assays. However, addition of EDTA or spermine to the growth medium slowed down or even prevented autolysis, and allowed measurement of growth by extinction determinations (Table 2).

Table 2. *Prevention of autolysis of isolate 544 in complete medium by spermine and EDTA*

Isolate 544 was grown in the complete medium. When EDTA was used it was added to the medium before autoclaving it; spermine was added aseptically after autoclaving.

Additions to growth medium ($\mu g./ml.$)	Incubation time (hr)	Vitamin B_{12} ($m\mu g./ml.$)		
		0.025	0.050	0.100
		O.D. reading at 590 $m\mu$		
None	42	0.16	0.32	0.57
	68	0.08	0.13	0.24
Spermine (8.5)	42	0.20	0.31	0.53
	68	0.20	0.45	0.80
EDTA (5.0)	42	0.17	0.35	0.55
	68	0.29	0.50	0.79

Vitamin requirements. Growth experiments showed that isolate 544 required vitamin B_{12} and thiamine. The quantitative response to these vitamins is shown in Fig. 1*a, b*. Thiamine could not be replaced by the pyrimidine moiety of the vitamin (2-methyl-4-5-amino-methylpyrimidine) at concentrations up to 40 $\mu g./ml.$

Functions of vitamin B_{12} . The vitamin requirements of several *Arthrobacter* strains could not be satisfied by thymidine or methionine (Lochhead & Burton, 1955; Ford & Hutner, 1957). Furthermore, Goldberg *et al.* (1957) found no sparing of vitamin B_{12} by methionine. Our studies with isolate 544 likewise showed that vitamin B_{12} could not be replaced by methionine, thymidine or by acid and alkaline hydrolysates of DNA or RNA. However, we found that methionine (20–30 $\mu g./ml.$) had a sparing effect on the vitamin B_{12} requirement in the basal medium (Fig. 2). This effect indicated the possibility of a vitamin B_{12} function in the biosynthesis of methionine. The effect of vitamin B_{12} on the methylation of homocysteine was therefore tested with vitamin B_{12} -deficient suspensions of isolate 544 (Table 3). As can be seen, addition of vitamin B_{12} caused a three-fold increase in the amount of methionine formed from homocysteine and serine. Addition of vitamin B_{12} to suspensions grown with optimal concentrations of vitamin B_{12} had no effect on the synthesis of methionine.

Effect of vitamin B_{12} on respiration. Although methionine spared vitamin B_{12} , it did not replace the vitamin for isolate 544. Additional functions of vitamin B_{12} in this

Fig. 1. Quantitative response of isolate 544 to thiamine and vitamin B₁₂. (a) Response to vitamin B₁₂, using the assay procedure described (see Methods); (b) response to graded amounts of thiamine in the thiamineless medium, supplemented with vitamin B₁₂ (0.5 mμg./ml.). The inoculum was taken from the same medium supplemented with thiamine (0.4 mμg./ml.) after incubation for 48 hr. Thiamine HCl was added aseptically after autoclaving the medium. Cultures were incubated for 48 hr at 30°.

Fig. 2. Effect of DL-methionine on growth of isolate 544 in the presence of increasing concentrations of vitamin B₁₂. Broken line, basal medium (see Table 1); solid line, basal medium + DL-methionine 20 μg./ml. Incubation for 60 hr at 30°.

Fig. 3. Effect of vitamin B₁₂ on the endogenous respiration and the oxidation of various substrates by isolate 544. Solid lines, in presence of vitamin B₁₂; broken lines, without vitamin B₁₂.

Table 3. Effect of vitamin B₁₂ on the methylation of homocysteine by suspensions of isolate 544

Isolate 544 was incubated for 60 hr in basal medium (without methionine), supplemented with a suboptimal concentration of vitamin B₁₂ (0.075 mμg./ml.). Organisms were collected by centrifugation, washed twice with distilled water and incubated (equiv. 0.6 mg. dry wt organism/ml.) for 12 hr at 30° with shaking. Incubation mixtures contained mM: DL-homocysteine, 5; L-serine, 5; glucose, 25; vitamin B₁₂, 10 nM. Incubation was terminated by boiling the reaction mixtures for 5 min. and the organisms removed by centrifugation. Methionine in the supernatant fluids was assayed microbiologically with *Streptococcus equinus* (ATCC 7881) by using Difco methionine assay medium.

Additions to reaction mixture			L-methionine formed (μmoles/mg. dry wt organisms)
Homocysteine	Serine	Vitamin B ₁₂	
+	+	+	0.65
+	+	—	0.20
+	—	+	0.38
—	+	+	0.10

organism have not been clearly defined. However, it was found that vitamin B₁₂-deficient isolate 544 organisms were unable to oxidize propionate unless supplied with vitamin B₁₂. The effect of vitamin B₁₂ on the respiration with glucose, various organic acids and amino acids was therefore tested. The endogenous respiration and the respiration with glucose, pyruvate, butyrate, leucine and lysine was only slightly

stimulated (5–20 %) by vitamin B₁₂. On the other hand, addition of the vitamin markedly increased (30–80 %) the oxygen uptake in presence of propionate, *n*-valerate, isovalerate, isoleucine and valine; the oxidation of Tween 80, acetate and succinate was stimulated to a lesser extent (25–45 %). The effect of vitamin B₁₂ on the respiration of isolate 544 in the presence of some of these compounds is shown in Fig. 3.

Growth response to vitamin B₁₂ analogues

The growth response of isolate 544 to various vitamin B₁₂ derivatives was tested by using the tube assay. Table 4 compares the activities of some naturally occurring and some biosynthetically prepared analogues of vitamin B₁₂ for isolate 544, *Ochromonas*

Table 4. Activity for various micro-organisms of some naturally occurring and biosynthetically prepared analogues of vitamin B₁₂

The activity of vitamin B₁₂ was taken as 100. Activities of the various B₁₂ analogues are means of 3–5 different assays. All compounds were tested over a wide range of concentrations (10–100-fold). Factors B, Z₂ and Z₃ were tested at concentrations up to 0.1 µg./ml.; the anilide and ethylamide of the monocarboxylic acid of vitamin B₁₂ were tested at concentrations up to 0.5 µg./ml. Considerable fluctuations in activities were noted with some of the B₁₂ analogues (benzimidazolylcobamide cyanide) even under carefully standardized conditions. Limit values given for the activities of the anilide and ethylamide analogues for isolate 544 were obtained after incubation times of 70 and 84 hr, respectively. Values for *O. malhamensis* were obtained after incubation for 96 hr.

Analogue	Test organism		
	Isolate 544 (<i>Arthrobacter</i> sp.)	<i>Ochromonas</i> <i>malhamensis</i>	<i>Escherichia</i> <i>coli</i> 7M
	Activity (%)		
α-(5,6-Dimethylbenzimidazolyl)-cobamide cyanide (vitamin B ₁₂)	100	100	100
α-(Benzimidazolyl)cobamide cyanide	1.5–3	57–90	130–150
α-(5-Methylbenzimidazolyl)cobamide cyanide	9	72	100
α-(5-Hydroxybenzimidazolyl)cobamide cyanide (vitamin B ₁₂ III)	8	32	30
α-(Adenyl)cobamide cyanide (pseudovitamin B ₁₂)	0.05	0.02	60
α-(2-Methyladenyl)cobamide cyanide (factor A)	0.5	0.3	70
α-(2-Methylmercaptopadenyl)cobamide cyanide (factor F)	6	5	100
Cobinamide (factor B)	0.005	0.005	42
Factor Z ₂	0.4	0.1	60
Factor Z ₃	0.1	0.08	10
Anilide analogue of vitamin B ₁₂	0.04–0.1	0.025	0.025
Ethylamide analogue of vitamin B ₁₂	0.2–0.8	0.005	0.02

malhamensis and *Escherichia coli*; the activity of vitamin B₁₂ was taken as 100 %. The pattern of response of isolate 544 was similar to that of *O. malhamensis* (Bernhauer, 1956; Peterson, Hall & Bird, 1956; Coates & Kon, 1957). Isolate 544 responded neither to cobinamide (Factor B) nor to factors Z₂ and Z₃, which probably also lack the nucleotide component (Neujahr, 1956); α-(adenyl)cobamide cyanide (pseudovitamin B₁₂) and α-(2-methyladenyl) cobamide (Factor A) had little activity for both organisms (less than 1 %). Of the naturally occurring analogues, isolate 544 responded only to α-(5-hydroxybenzimidazolyl)cobamide cyanide (vitamin B₁₂ III and to α-(2-mer-

captoadenyl)cobamide cyanide (Factor F) with an activity of 8 % and 6 %, respectively. However, the response of isolate 544 to α -(5-hydroxybenzimidazolyl)cobamide cyanide was considerably weaker (8 %) compared to that of *O. malhamensis* (32 %).

The biosynthetically prepared benziminazole analogues tested (benzimidazolyl cobamide cyanide and 5-methylbenzimidazolyl cobamide cyanide) which are almost fully active for *Ochromonas malhamensis* had only a slight activity for isolate 544. The anilide and ethylamide of the monocarboxylic acids of vitamin B₁₂ are practically inactive as vitamin B₁₂ substitutes for *Escherichia coli* and *O. malhamensis*. Both analogues exhibited some growth-promoting activity for isolate 544; the activity of the ethylamide being about 8 times higher than that of the anilide. However, on prolonged incubation, *O. malhamensis* also showed some growth on the anilide.

Table 5. *Effect of substituted vitamin B₁₂ amides on the growth of isolate 544*

The cultures were incubated for 72 hr at 30°

Analogue added (m μ g./ml.)	Vitamin B ₁₂ (m μ g./ml.)				
	0	0.25	0.1	0.4	0.8
	O.D. scale reading at 590 m μ				
None	0	0.21	0.90	1.42	1.50
Anilide					
10	0.15	—	—	—	—
1 $\times$ 10 ²	0.65	0.97	1.11	1.30	1.35
1 $\times$ 10 ³	0.76	0.86	0.90	1.06	1.28
1 $\times$ 10 ⁴	0.03	0.05	0.05	0.06	0.06
Ethylamide					
10	0.76	—	—	—	—
1 $\times$ 10 ²	1.24	1.10	1.28	1.44	1.50
1 $\times$ 10 ³	1.18	1.12	1.16	1.20	1.49
1 $\times$ 10 ⁴	1.04	1.09	1.06	1.04	1.08

Both the ethylamide and the anilide of vitamin B₁₂ are growth inhibitors for *Ochromonas malhamensis* and *Escherichia coli* (Ford, 1959; Baker *et al.* 1960; Smith, 1960). With isolate 544 it was found (Table 5), that the anilide (1 $\times$ 10⁴ m μ g./ml.) completely inhibited growth in the presence of up to 0.8 m μ g./ml. vitamin B₁₂. At this concentration of the analogue and increasing concentrations of vitamin B₁₂, the 50 % inhibition index was calculated to be 2500. On prolonged incubation (96 hr), the inhibition index for *O. malhamensis* was about 3000, and for *E. coli* 7M, which is more sensitive to cyanocobalamin, the inhibition index was about 10,000.

Use of isolate 544 for assay of vitamin B₁₂

Isolate 544 gives homogeneous growth in liquid media and grows well on suitable solid media. The strain is thus suitable for tube and plate assays of vitamin B₁₂.

Procedure of tube assay. The assay medium (Table 4), at 5/4 concentration was dispensed in 4 ml. amounts into 19 $\times$ 150 mm. test-tubes, and the volume made up to 5 ml. with either water or material to be tested.

Standard curve. A series of known concentrations of vitamin B₁₂ (standard curve) was included in each assay. One ml. samples of appropriate dilutions of a sterile stock solution of vitamin B₁₂ (1.5 m μ g./ml.) were added to give final concentrations of

0.00625, 0.0125, 0.025, 0.050, 0.1, 0.15, 0.2 and 0.3 $\mu\text{g./ml.}$ Tubes without added vitamin B₁₂ were included as blanks.

Inoculum. The organism was maintained on solid assay medium supplemented with vitamin B₁₂ (1 $\mu\text{g./ml.}$) and agar (1.5 %). Growth from a 48 to 72 hr slope was removed with a wire loop and disposed in 10 ml. assay medium (in a 50 ml. Erlenmeyer flask) supplemented with vitamin B₁₂ (0.1 $\mu\text{g./ml.}$) to obtain a barely turbid suspension. The culture was incubated with shaking for 48 hr at 30°; 0.1 ml. of this culture was used to inoculate each assay tube.

Assay. Vitamin B₁₂ was extracted from the material (adjusted to pH 4.5 with HCl) to be tested according to the procedure described previously (Aronovitch & Grossowicz, 1958) except for the acetate buffer and NaCN which were used at one-tenth of

Table 6. *Comparative assays by two organisms, isolate 544 and Escherichia coli 7M of vitamin B₁₂ in various materials*

Each sample was tested twice over a wide range of concentrations. Vitamin B₁₂ values of liver and soil extract are expressed as $\mu\text{g./g.}$, vitamin B₁₂ values of serum are given as $\mu\text{g./ml.}$

Material assayed	Test organism	
	Isolate 544	<i>E. coli</i> 7M
	Vitamin B ₁₂ conc. ($\mu\text{g.}$)	
Human serum normal, pooled	0.49	0.55
Human myelogenous leukaemia serum, pooled	2.20	2.10
Rabbit serum	10.90	12.80
Human liver (myelogenous leukaemia)	61.00	67.00
Rat liver	360.00	430.00
Soil extract	1.13	1.75

the previous concentration because of their adverse effect on the growth of isolate 544. One ml. samples of appropriate dilutions of the extracts to be tested were added to 4 ml. medium and sterilized by autoclaving for 15 min. at 115°. The assay tubes were incubated at 30° on a shaker for 48 hr. Growth was measured turbidimetrically. Figure 1 shows the response to graded amounts of vitamin B₁₂. The growth response was linear over a wide range of concentrations (0.005–0.1 $\mu\text{g./ml.}$).

The assay medium when supplemented with an optimal concentration of vitamin B₁₂ was fully adequate for the growth of isolate 544; addition of defined growth factors and complex natural materials increased neither the growth rate nor the maximum yield of organisms; among the materials tested were various B vitamins, purine and pyrimidine mixtures, acid and alkaline hydrolysates of DNA and RNA and extracts of yeast, liver and soil.

Comparative assays of vitamin B₁₂ activity in various natural materials were made with isolate 544, *Ochromonas malhamensis* and *Escherichia coli* 7M. The vitamin B₁₂ values obtained with isolate 544 were similar to those obtained with *O. malhamensis*, and in general somewhat lower than those found with *E. coli*. Samples of human serum and liver gave similar values with all three bioassays. On the other hand, soil extracts showed significantly lower vitamin B₁₂ activity (65 %) when assayed with isolate 544 than with *E. coli* 7M (Table 6).

DISCUSSION

The morphological characteristics of isolate 544 fit the genus *Arthrobacter* as described by Conn & Dimmick (1947). However, biochemically, isolate 544 differs from the several species described in *Bergey's Manual* (1957). The vitamin requirements of isolate 544 are similar to those of *Arthrobacter duodecalis* (Lochhead, 1958), and to the organisms belonging to type II (Lochhead & Burton, 1955). However, isolate 544 differs from the latter in the production of acid from glucose, and is able to utilize nitrate and ammonium salts as sole nitrogen sources. Isolate 544 was therefore tentatively classified as *Arthrobacter* sp.

Although methionine was ineffective in replacing vitamin to B₁₂ for isolate 544, it spared the vitamin for growth. Addition of vitamin to B₁₂-deficient cell suspensions stimulated the methylation of homocysteine. A similar stimulatory effect was found with crude extracts prepared from these organisms. The sparing effect, as well as the enhancement of the biosynthesis of methionine by vitamin B₁₂, indicate that in this organism the vitamin is involved in the methylation of homocysteine. Isolate 544 differs in this respect from the *Arthrobacter* strains studied by Goldberg *et al.* (1957). A similar effect of vitamin B₁₂ on the biosynthesis of methionine has been shown in *Escherichia coli* (Gibson & Woods, 1960), and indicated in *Ochromonas malhamensis* (Johnson, Holdsworth, Porter & Kon, 1957; Dalal, Rege & Sreenivasan, 1961).

Other functions of vitamin B₁₂ in isolate 544 have not been well defined. The effect of the vitamin on the respiration of vitamin B₁₂-deficient organisms indicated that vitamin B₁₂ plays a role in the oxidation of odd-numbered and branched-chain organic acids. Vitamin B₁₂-deficient organisms of isolate 544 depended on the vitamin for oxidation of propionate. A similar effect of vitamin B₁₂ was found with vitamin B₁₂-deficient cells of *Ochromonas malhamensis* (Marchesi & Lajtha, 1961; Arnstein & White, 1962), and with two *Flavobacterium* strains (Ayers, 1962*a, b*). With isolate 544 and with one of the *Flavobacterium* strains, vitamin B₁₂ also stimulated the oxidation of some other odd-numbered organic acids as well as the oxidation of certain amino acids (valine, isoleucine), while the endogenous respiration and the oxidation of other amino acids (leucine, lysine) were only slightly affected. It seems likely therefore that all these substrates lead to the production of a common intermediate, as proposed by Ayers (1962*a*). The common intermediate, probably methylmalonyl-CoA, is isomerized to succinyl-CoA in a vitamin B₁₂-dependent reaction similar to that shown for other bacterial species (Stadtman, Overath, Eggerer & Lynen, 1960). Stimulation of the endogenous respiration by vitamin B₁₂ suggests that the endogenous substrate consists, at least in part, of compounds that yield methylmalonyl-CoA as an intermediate.

Of the naturally occurring B₁₂-analogues tested, isolate 544, like *Ochromonas malhamensis* and higher animals, utilizes only vitamin B₁₂III and factor F. The response of isolate 544 to vitamin B₁₂III is less than 10% of its response to cyanocobalamin, whereas the response of *O. malhamensis* is 30–70% (Kon & Pawelkiewicz, 1958). The sensitivity of isolate 544 to cyanocobalamin is similar to that of *O. malhamensis*. However, isolate 544 is faster growing and easier to maintain than *O. malhamensis* and unlike the latter grows well on solid media and may therefore be used in the convenient pad plate-assay. Only small amounts of vitamin B₁₂ are accumulated in isolate 544 organisms grown on optimal concentrations of the vitamin; large inocula may

therefore be used without washing. The medium used is suitable for the assay of vitamin B₁₂ in crude extracts; the response to the vitamin was not affected by the addition of defined growth factors or yeast extract. Isolate 544 has proved to be very stable; vitamin B₁₂-independent mutants have not been observed. Under proper aeration only minor variations in response were encountered in repeated assays. Isolate 544 seems therefore to be a suitable organism for use in the specific assay of cyanocobalamin in crude extracts.

The anilide and the ethylamide of the monocarboxylic acids of vitamin B₁₂ acted as vitamin B₁₂ antagonists for isolate 544. The growth inhibition caused by these analogues was annulled by vitamin B₁₂, indicating a competitive type of inhibition. Both analogues have vitamin B₁₂ activity at the concentrations used for inhibition studies, but growth on the analogues was slower than on vitamin B₁₂. The relative antagonistic activity was tested after a prolonged period of incubation (72 hr); under these conditions the anilide was a more active analogue than the ethylamide. A similar order of activities has been found for the growing chick (Coates, Davies & Harrison, 1960). The two amides have a reversed order of activities towards *O. malhamensis* and *E. coli* (Ford, 1959; Baker *et al.* 1960; Smith, 1960).

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